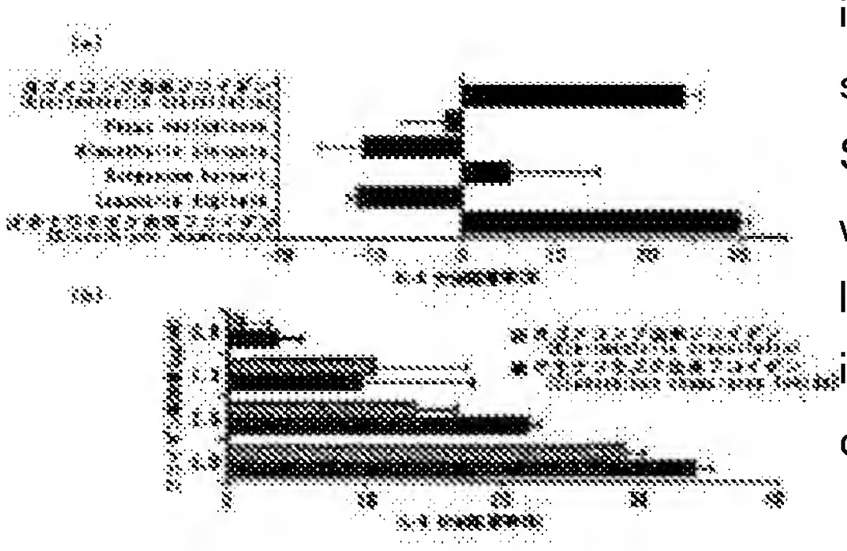
(54) **PREVENTIVE AND THERAPEUTIC AGENT OF INFLAMMATORY INTESTINAL DISEASE**



(57)Abstract:
PROBLEM TO BE SOLVED: To obtain a preventive and therapeutic agent for inflammatory intestinal diseases containing a safe material as an effective ingredient.
SOLUTION: Fucoidan or an extract of sea weeds such as Nematocystus decipiens and the like containing fucoidan is used as an effective ingredient of the inflammatory intestinal diseases.

CLAIMS

[Claim(s)]
[Claim 1]An inflammatory-bowel-disease prevention treating agent which makes a fucoidan an active principle.
[Claim 2]The inflammatory-bowel-disease prevention treating agent according to claim 1 which makes a seaweed extract containing a fucoidan an active principle.
[Claim 3]The inflammatory-bowel-disease prevention treating agent according to

claim 2 which makes the Nemacystus extract containing a fucoidan an active principle.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention]This invention relates to a safe inflammatory-bowel-disease prevention treating agent.
[0002]
[Description of the Prior Art]Inflammatory bowel disease (Inflammatory Bowel Disease:IBD), such as ulcerative colitis and Crohn's disease, is intestinal diseases by which the cause is not yet clarified.
Establishment of various therapies and preventing methods is made into pressing need now.
It is the feature of this lesion that recovery and a recurrence of the enteritis lesion over a long period of time in inflammatory bowel disease are repeated.
At present, it cannot but depend on a therapy at the medication method over a long period of time.

[0003]On the other hand, by our old research, animal IBD either Homo sapiens IBD or a model. The STAT(Signal Transducer and Activator of Transcription)-3 phosphorylation response is found out accelerating in the intestinal-mucosa epithelial cells of a lesion part. As a molecule in connection with phosphorylation of STAT-3 molecule, interleukin (IL)-6, a leukemia proliferation inhibiting factor (LIF), the

oncostatin M (OSM), etc. are known.

[0004]In an IBD model animal and Homo sapiens IBD, it is also clear that the IL-6 production by an intestinal epithelium cell is accelerating. Therefore, phosphorylation of STAT-3 of an intestinal epithelium cell which an IBD model animal and Homo sapiens IBD are permitted has a high possibility of originating in IL-6 produced in an intestinal epithelium cell in connection with inflammation.

[0005]IL-6 forms soluble IL-6 receptor (IL-6R) and complex which are secreted by an intestinal epithelium cell or monocyte, and the macrophage at the time of an inflammatory reaction. 130 molecules of gp(s) of the membrane glycoprotein which is a signal transfer chain of IL-6 receptor revealed into almost all cells including a non-lymphoid cell meet with complex-ized IL-6/IL-6R, and transmit a signal to intracellular.

[0006]Therefore, IL-6/IL-6R complex-ized transmit a signal also to the cell lineage which has not revealed IL-6R by combining with 130 molecules of gp(s), and derives an inflammatory reaction. From the above thing, control of an IL-6/STAT-3 phosphorylation response system attracts attention as an inhibition method of various inflammatory diseases now.

[0007]

[Problem to be solved by the invention]In inflammatory bowel disease, side effects pose a problem under the therapy by the medication method over a long period of time like before. Then, it is thought that development of the restrainning of the symptoms using safe foodstuffs with few side effects is very useful for the therapy of inflammatory bowel disease.

[0008]Like the above, the control of a STAT-3 phosphorylation response system as which sthenia is regarded also in the lesion part intestinal epithelium cell of inflammatory bowel disease can also expect control of the inflammatory lesion of inflammatory bowel disease, and can consider the depressor effect of IL-6 production as the control method of this STAT-3 phosphorylation response system.

[0009]Therefore, if prevention and a treating agent which uses a safe substance as the main ingredients are obtained while it has IL-6 production depressor effect, it will also become possible to prevent or treat inflammatory bowel disease effectively and safely.

[0010]The purpose of this invention is to provide an inflammatory-bowel-disease prevention treating agent which makes a safe substance an active principle in view of the above-mentioned problem.

[0011]

[Means for solving problem]In order to attain the above-mentioned purpose, an inflammatory-bowel-disease prevention treating agent concerning the invention according to claim 1 makes a fucoidan an active principle.

[0012]An inflammatory-bowel-disease prevention treating agent concerning the invention according to claim 2 makes a seaweed extract containing a fucoidan an active principle in the inflammatory-bowel-disease prevention treating agent according to claim 1.

[0013]An inflammatory-bowel-disease prevention treating agent concerning the invention according to claim 3 makes the Nemacystus extract containing a fucoidan an active principle in the inflammatory-bowel-disease prevention treating agent according to claim 2.

[0014]Lipopolysaccharide (LPS) is a cell wall component of Gram negative bacterium, such as Bacteroides and Escherichia coli. In an inflammatory-bowel-disease patient, it is known well that a total of the Bacteroides bacillus in an intestinal bacterial flora is increasing, and it is also reported that superfluous growth of the Bacteroides kind hates enteritis of transgenic rat HLA-B27. In a mouse in which a gene of an LPS receptor (TLR4) of cell surface was destroyed, it is reported that colitis is also slighter than a control mouse.

[0015]Therefore, an LPS signal is an important element in hatred[the onset /]-izing of inflammatory bowel disease. Direct influence of a bacteria ingredient like LPS is carried out to the intestines surface, and it is thought that an immune response in an intestinal epithelium cell is induced. That is, an LPS signal causes production of IL-6 in an intestinal epithelium cell, and is considered to generate colitis. Therefore, a possibility that removal of these signals from an intestinal epithelium cell will contribute to an improvement of enteritis is high.

[0016]Much biological effectiveness, like this invention prevents fixing disturbance of Helicobacter Pylori to human stomach [this invention persons] cell lineage, and infection to a human cell line of a virus of a certain kind is reported, Can obtain from marine algae etc. and its attention is paid to a fucoidan with high safety as which neither toxicity nor a stimulus is regarded by the polysaccharide, A place which examined IL-6 production depressor effect of various seaweed origin fucoidans in a mouse intestinal epithelium cell system by which colitis was induced by lipopolysaccharide (LPS: lipopolysaccharide) stimulus, It finds out that a thing with IL-6 production depressor effect is in said fucoidan, and further, by this, it finds out becoming an active principle of prevention and a treating agent to inflammatory bowel disease, and results in this invention.

[0017]Especially, it found out that the IL-6 production depressor effect of a fucoidan of the Nemacystus origin, especially a fucoidan of Cladosiphon okamuranus origin was high compared with a fucoidan of other substance origin, and they were desirable as an active principle of an inflammatory-bowel-disease prevention treating agent.
[0018]A fucoidan is sulfuration polysaccharide which is abundantly contained in brown algae, sea cucumber body walls, etc., such as Nemacystus, a Fucus vesiculosus, wakame seaweed, and which mainly consists of fucoses. Therefore, it can obtain from various food with high safety eaten for many years, and high safety can also be secured to the prevention treating agent of the inflammatory bowel disease which made the extract containing a fucoidan or a fucoidan the active principle.
[0019]
[Mode for carrying out the invention]as the fucoidan used for this invention -- Cladoshipon okamuranus Tokida of brown algae -- the so-called fucoidan of Cladosiphon okamuranus origin. Kjellmaniella crassifolia -- the so-called fucoidan of GAGOME Fucus-vesiculosus origin. the fucoidan of Himmanthalia elongata origin, the fucoidan of Sargassum horneri origin, the fucoidan of Lamanaria digtata origin, and Fucus vesiculosus -- the so-called fucoidan of fucus origin, etc. are mentioned.
[0020]The method used from the former may be used as the method of preparation of the fucoidan from each seaweed. For example, ** acid extraction method and ** hot water extract method which are illustrated below are typical.
[0021]** making the 1-3-times the amount water of the wet weight suspend acid extraction method seaweed, and adding an acetic acid solution or dilute hydrochloric acid -- pH 2-4 -- adjust to pH 2-3 desirably. Subsequently, after heating at 80-100 ** desirably and eluting not less than about 50 ** of fucoidans, it centrifuges, and except for a sediment, supernatant liquid is neutralized by sodium hydroxide and an extract is obtained. A fucoidan with high purity is obtained by performing ultrafiltration, dialysis, etc. further if needed and freeze-drying except for the impurity of low molecular weight.
[0022]** Make the 1-3-times the amount water of the wet weight suspend hot extraction method seaweed, and heat at 100 ** for about 10 minutes - 1 hour. Subsequently, the extract which centrifuges and contains a fucoidan except for a sediment is obtained. The alginic acid which adds a calcium chloride or barium acetate to supernatant liquid, and precipitates as occasion demands is excluded. After dialyzing and removing the impurity of low molecular weight, if it freeze-dries, a fucoidan with high purity can be obtained.
[0023]In this invention, it is preferred to use a fucoidan with high purity except the

impurity of low molecular weight as an active principle of an inflammatory-bowel-disease prevention treating agent. In the arbitrary stages of removing the impurities (water-soluble materials, salts, etc.) of low molecular weight, especially the thing for which ion exchange treatment is performed and the sulfate group of a fucoidan is changed into a free acid form or an alkaline-metal-salt form is preferred. The neutralization processing following it, etc. can perform ion exchange treatment, for example with either electrodialysis, the acid cleaning which uses ultrafiltration membrane, ion exchange resin treatment or these processings. these processings -- each -- a law -- if what is necessary is just to carry out in accordance with a method and alkali metal hydroxide, such as mosquito nature soda and mosquito nature potash, neutralizes the fucoidan after ion exchange treatment, alkaline metal salt can be obtained.

[0024]Since the safety of the fucoidan whose inflammatory-bowel-disease prevention treating agent of this invention is the active principle is established, Even if it administers orally then, it is satisfactory, but if needed An excipient, a binding material, As long as it does not check the effect of a fucoidan, it may add suitably, and disintegrator, lubricant, coating, an emulsifier, a dispersing agent, a solvent, a stabilizing agent, etc. may manufacture medicine and use it for a tablet, a granule, powder medicine, powders, a capsule, etc. the dose of a moderate fucoidan -- in general -- a 50mg-3g/person/day -- desirable -- -- it is a 100mg-1g/person/day.

[0025]The inflammatory-bowel-disease prevention treating agent of this invention can also be made to take in daily with the form of the prevention foodstuffs added not only to the internal use as a treating agent but to arbitrary ingesta. Under the present circumstances, the fucoidan of an active principle can manufacture and provide the prevention foodstuffs of inflammatory bowel disease simple by low cost more, if not only a high grade thing but the fucoidan content extract extracted from marine algae at the easy process is used.

[0026]

[Working example]Although working example is given and this invention is explained in detail hereafter, this invention is not limited to these. In subsequent working example, the fucoidan of the high grade except the impurity of low molecular weight prepared with the above-mentioned conventional method and a commercial fucoidan comparable as this shall be used.

[0027](1) Material Cladosiphon okamuranus (Cladoshipon okamuranus Tokida) purchases what was cultivated in Okinawa as foodstuffs preserved in salt from Tropical Technology Center, Other seaweed (Kjellmaniella crassifolia, Himmanthalia

elongata, Sargassum horneri, Lamanaria digitata) is supplied by SCETI as a dried food, and from these each seaweed. A fucoidan of a high grade was extracted with the above-mentioned conventional method. A fucoidan of Fucus vesiculosus origin was purchased from a sigma company.

[0028](2) all the data shown in working example after data analysis -- as average value**SE (statistical error) -- a table -- the bottom. P numerical value smaller than 0.05 presupposes statistically that it is significant.

[0029]In working example 1 this example, mouse colon cancer cell CMT-93 is investigated for IL-6 production depressor effect of various fucoidans in IL-6 production after an LPS stimulus. CMT-93 [in beginning this experiment] to an LPS stimulus of concentration of versatility first IL-6 production of a cell strain was investigated.

[0030](A) IL-6 production-amount mouse colon cancer cell strain CMT-93 in LPS stimulus CMT-93 cell was purchased from ATCC. LPS used a thing of E.coli origin purchased from a sigma company. CMT-93 10%FCS/10/2-ME/non-essential aminoacid/DMEM culture medium is used for a cell, It cultivated under LPS addition conditions of concentration of LPS additive-free or versatility under conditions of 37 ** and 5%CO_2 for 72 hours. Culture medium was collected after culture and it saved at -84 **. IL-6 production amount was measured by the ELISA method using anti-IL-6mAbs (a clone: MP5-20F3, MP5-32C11) of a mouse purchased from BDPharMingen.

[0031]As a result, it is CMP-93 as shown in drawing 1. IL-6 production amount in a cell reached the maximum, when LPS was 5-10micrometers/ml, and it was saturation in ml in 10 microg /.

[0032](B) LPS stimulus CMP-93 It is LPS stimulus CMP-93 about the fucoidan of IL-6 production depressor effect aforementioned each seaweed origin of the fucoidan to a cell. It was investigated whether the IL-6 production in a cell could be controlled. Namely, CMT-93 which added 1 microg/ml various fucoidans 10microg/mlLPS was added to the cell culture line, and it cultivated for 72 hours. Culture medium was collected after culture and IL-6 production amount was measured by the ELISA method like the above.

[0033]As a result, as shown in drawing 2 (a), the fucoidan of Cladosiphon okamuranus (Cladoshipon okamuranus Tokida) origin and the fucoidan of GAGOME Fucus-vesiculosus (Kjellmaniella crassifolia) origin, LPS stimulus CMP-93 The IL-6 production in a cell was controlled. However, the fucoidan of other Fucus vesiculosus, Himmanthalia elongata, Sargassum horneri, and Lamanaria digitata origin did not

control IL-6 production.
[0034]Next, LPS stimulus CMP-93 in fucoidan addition concentration which is different about said Cladosiphon okamuranus origin fucoidan and GAGOME Fucus-vesiculosus origin fucoidan controlling IL-6 production turned out to be The IL-6 production depressor effect in the cell was investigated. Measurement of IL-6 quantity by the cell culture and the ELISA method is the same as that of the above.
[0035]As a result, as shown in drawing 2 (b), also in which fucoidan, IL-6 production amount was controlled depending on the addition concentration.
[0036]In working example 2 this example, the improvement effect of the fucoidan to the mouse chronicity colitis in in vivo was investigated.
[0037](C) In animal this example, the female Balb/c mouse (8-week old) (it purchases from a Japanese clear research institute company) was used. These mice are bred under the SPF (Specific pathogen-free) environment during an experiment.
[0038](D) Dextran sulfate natrium (DSS) performed induction of the chronic colitis to the induction Balb/c mouse of chronic DSS colitis. After medicating a 10-week old mouse with DSS (made by molecular weight 40 kDa:ICN) water as drinking water for seven days 4%, chronic colitis was induced in a total of four cycles by making the method of making a resting phase ten days which will continue into one cycle.
[0039](E) the fucoidan effect standard mouse feed (MF) in mouse chronicity colitis in in vivo, and fucoidan content (0.05%w/w) MF feed of Cladosiphon okamuranus (Cladoshipon okamuranus Tokida) origin. Or fucoidan content (0.05%w/w) MF feed of fucus (Fucus vesiculosus) origin, Like the above, to a Balb/c mouse bred by **********, colitis was induced with drinking water containing DSS, and various disease parameters analyzed the fucoidan effect in colitis to it.
[0040](1) Score conversion was carried out with a step number value of 0-4, respectively by making distinctive alteration of a disease of weight, diarrhea, and facilities occult blood into a parameter, and evaluations of an enteritis disease evaluation enteritis disease are a numerical value of each parameter, and the numerical total, and were checked as reflection of change in clinical progress of a DSS colitis mouse.
[0041]As shown in drawing 3 (a), in a Balb/c mouse in which Cladosiphon okamuranus fucoidan content feed was given to a case where standard feed or fucus origin fucoidan content feed is given, numerical value of a result was [a score of all the parameters] low.
[0042](2) The length of the large intestine from an intestines organization head evaluation cecum to the anus was measured as a severity parameter of DSS colitis.

[0043]As a result, as shown in drawing 3 (b), the colitis induction Balb/c mouse of the intestines organization head in which Cladosiphon okamuranus origin fucoidan content feed was given was more nearly intentionally [than the thing of a mouse which was able to give standard feed] longer. In the colitis induction Balb/c mouse which was able to give standard feed and fucus origin fucoidan content feed, the length of the large intestine was short for serious-illness-izing of enteritis.
[0044](3) In order to check the result of the myeloperoxidase (MPO) measurement above, the MPO activity of the intestines organization was compared among 3 groups. Measurement of MPO activity was performed in the following procedures. That is, first, each mouse large intestine organization was crushed with the polyTRON homogenizer in hexadecyl trimethylammonium bromide (made by sigma company) buffer solution, and after carrying out sonication of the suspension in Hikami, centrifugal separation for 30 minutes was performed at 15000 rpm. Each supernatant liquid was mixed to 0.167mg/ml O-dianisidine hydronalium chloride (made by a sigma company), and the enzyme substrate buffer solution which contains hydrogen peroxide 0.0005%, change of the absorbance in 405 nm wavelengths was measured, respectively, and MPO activity (U/g proten) was searched for.
[0045]As a result, as shown in drawing 4, in the mouse which was able to give Cladosiphon okamuranus origin fucoidan content feed, intestines organization MPO activity was lower than the mouse which was able to give fucus origin fucoidan content feed or standard feed.
[0046]It became clear from the above result to equip the fucoidan of Cladosiphon okamuranus origin with the effect of improving the chronic colitis in a mouse.
[0047]In working example 3 this example, the immunological feature of the Cladosiphon okamuranus origin fucoidan was investigated.
[0048](F) The lymphocyte production and the flow-cytometry control Balb/c mouse, and the colitis induction Balb/c mouse derived by DSS compared the phenotype of large intestine lamina propria mucosae (IL-LPLs).
[0049]It incubated shaking the piece of the large intestine cut into every 1 cm 37 ** and twice [every / a for / 15 minutes] in the balanced saline (HBSS) of Hanks containing 0.45mMDTT and 2mMEDTA. The piece of the large intestine which remained after removal of the solution layer by a decantation 2.5% fetal calf serum (FCS) and 300 microg [/ml] collagenase (collagenase Yakult S; made by Yakult Honsha), With RPMI1640 culture medium containing 50 microg [/ml] deoxyribonuclease I (made by a sigma company), it incubated 37 **, and every [a for / 45 minutes] 3 times, shaking with CO_2 humidistat. Then, the cell lump was

suspended in ice-cooled 2.5%FCS / 10 mMHepes/RPMI liquid, and the nylon column was passed. The lymphocyte group was separated from the 44/100% boundary of the PARCOR density gradient (made by a sigma company). The obtained cell was dyed by mAbs to TCRbeta, CD4, CD45RB, CD69, or B220. The cell after dyeing was analyzed by the EpicsEL cell analyzer (made in Beckmann).

[0050]Based on these results, phenotype of IL-LPLs was compared among three groups of a colitis induction Balb/c mouse who were able to give standard feed and Cladosiphon okamuranus origin fucoidan content feed and fucus origin fucoidan content feed, respectively.

[0051]As a result, it is B220 as shown in drawing 5. A total of a positive B cell was intentionally lower than other groups' mouse in an Cladosiphon okamuranus origin fucoidan content feed administration mouse.

[0052](G) A lamina-propria-mucosae cell ($1.0x10^{6}$cell) from each colitis induction mouse of a cell culture and cytokine measurement aforementioned 3 group, It cultivated under a stimulus of anti-TCRbetamAb (H57-597 and 10 microg/(ml)) solid-phase-ized using 24 hole tissue culture plate in 10%FCS/10mM Hepes / 2-ME/RPMI culture medium, and anti-CD28mAb (37.51 and 1 microg/(ml)). The culture medium was collected after 72-hour culture, and it saved at -84 ** until it presented ELISA measurement.

[0053]Cytokine specific ELISA measurement was performed using the next antibody combination. Namely, anti-interferon (IFN) - It is gamma (clone: XMG1.2, R4-6A2) and the anti-interleukin 4 (IL-4) (clone: 11B11, BVD6-24G2), and these were all purchased from BDpharMingen. A bio-source international company, Genzyme, and the measurement kit purchased, more nearly respectively were used for IL-10 and measurement of TGF-beta 1.

[0054]In the large intestine lamina-propria-mucosae cell (IL-LPLs) stimulated with TCR beta/CD 28 antigen, As shown in drawing 6 (a) and (b), the production amount of inflammatory cytokine like IFN(interferon)-gamma and IL-6 was more nearly intentionally [than standard feed and a fucus origin fucoidan content feed administration mouse] low with the Cladosiphon okamuranus origin fucoidan content feed administration colitis induction mouse.

[0055]On the other hand, the production amount of TGF-beta 1 which is inflammation inhibitory cytokine was intentionally higher than standard feed and a fucus origin fucoidan content feed administration mouse in the Cladosiphon okamuranus origin fucoidan content feed administration colitis induction mouse (drawing 6 (e)). The production amount of IL-10 which is similarly inflammation inhibitory cytokine was

higher than the standard feed administration mouse in the Cladosiphon okamuranus origin fucoidan content feed administration colitis induction mouse (drawing 6 (d)). The production amount of IL-4 had the lowest standard feed administration mouse.

[0056](H) The crushing sample of the large intestine of the mouse of measurement aforementioned 3 group of the immunoglobulin G (IgG) in an intestines piece part was prepared like the above, and the production amount of intact IgG, IgG_1, and IgG_2 was measured by the specific sandwiches ELISA method.

[0057]As a result of measurement of IgG in the large intestine membrane in said three experiment groups, as shown in drawing 7, In the colitis induction Balb/c mouse which was able to give standard feed and fucus origin fucoidan content feed, respectively, an IgG total amount, IgG_1, and IgG_{2a} were increasing.

[0058]In working example 4 this example, the IL-6mRNA level on the large intestine epithelial cells of the colitis induction Balb/c mouse in which Cladosiphon okamuranus origin fucoidan content feed was given, It measured by the RT-PCR assay by considering as contrast a colitis non-inducing Balb/c mouse and the colitis induction Balb/c mouse which standard feed was able to give.

[0059]Large intestine epithelial cells were obtained from the colitis induction Balb/c mouse which was able to give a colitis non-inducing Balb/c mouse and standard feed, or Cladosiphon okamuranus origin fucoidan content feed, respectively.

[0060]All the RNA was prepared from these three mouse groups. The PCR reaction was performed for all the RNA of 1.0microg after the reverse transcription reaction using G3PDH, IL-6, TNF-alpha, and TLR-4 specific primers. After gel electrophoresis, the ethidium bromide dyed the PCR production thing and it was detected.

[0061]As a result, as shown in drawing 8, IL-6mRNA increased rapidly in the standard feed administration DSS colitis derivation Balb/c mouse. However, derivation of IL-6mRNA was controlled in the large intestine epithelial cells of an Cladosiphon okamuranus origin fucoidan content feed administration Balb/c mouse. TNF(tumor necrosis factor)-alpha and TLR-4 mRNA was also controlled in the large intestine epithelial cells of an Cladosiphon okamuranus origin fucoidan content feed administration Balb/c mouse.

[0062]In the above working example, the production depressant action of a fucoidan and IL-6 [in / especially the fucoidans of Cladosiphon okamuranus origin are in vivo and in vitro, and / large intestine epithelial cells] is shown, the improvement effect of a colitis lesion is also shown, and the high prevention curative effect of inflammatory bowel disease can be expected.

[0063]

[Effect of the Invention]According to this invention, high safety is established by the fucoidan which is an active principle, the enteritis lesion improvement effect based on IL-6 production depressant action is high, and offer of the safe and effective prevention treating agent of inflammatory bowel disease of the action mechanism is attained.